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How cyclodextrin glycosyltransferase works

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Document Version

Publisher's PDF, also known as Version of record

Publication date:

2000

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Uitdehaag, J. (2000). How cyclodextrin glycosyltransferase works: the atomic basis of catalysis and specificity in an enzyme from the alfa-amylase family. [Groningen]: University of Groningen.

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SUMMARY

Introduction

In chapter 1 an overview of the content of the thesis is given. In chapter 2 current data on telomerase in gynecological tumours, especially cervical and ovarian tumours, are reviewed. In more than 85% of gynecological malignant tumours unique overexpression of telomerase activity has been found, while benign tumours and normal tissues do not display or only weakly express telomerase activity. In cervical tissue, frequency of telomerase activity is related to grade of cervical intraepithelial neoplasia (CIN)/cervical cancer. In all gynecological tumours, the human telomerase reverse transcriptase (hTERT), the catalytic protein component of telomerase, is the important determinant for telomerase activity, as has also been observed in other malignancies¹¹⁻¹³. Additionally, several applications of determination of telomerase activity as a possible diagnostic tool and various ways for inhibition of telomerase are summarised.

T cell telomere length in HIV-1 infection: no evidence for increased CD4⁺ T cell turnover

Analysis of telomere length during a certain time period in cells with no or weak telomerase activity may serve as a marker for cell turnover. In human immunodeficiency virus type-1 (HIV-1) infection it has been suggested that CD4⁺ T cell (the T helper cells) turnover is increased, resulting in exhaustion of CD4⁺ T cell renewal and decline in CD4⁺ T cells. In chapter 3, telomere length is examined in T cells from HIV-1 infected patients. It reveals that telomere length decreases in CD8⁺ T cells (the cytotoxic T cells), but is stable in CD4⁺ T cells during the course of HIV-1 infection. This is not explained by differential telomerase activity. These data provide evidence that turnover in the course of HIV-1 infection may be increased considerably in CD8⁺ T cells, but not in CD4⁺ T cells. These results are compatible with CD4⁺ T cell decline in HIV-1 infection caused by interference with cell renewal.

Sequential telomere length measurement in breast cancer patients receiving standard or high-dose chemotherapy and stem cell transplantation

High-dose chemotherapy and peripheral blood stem cell transplantation may accelerate telomere length loss in hematopoietic stem cells. Data including pre- and post-treatment samples are lacking. Therefore, in chapter 4 leukocyte telomere length and telomerase activity are studied in breast cancer patients, before and after standard- or high-dose chemotherapy.

Peripheral blood hematologic parameters are decreased at both t_1 and t_2 (t_1 : 6 to 8 weeks after completion of chemo- and radiotherapy, and t_2 : 6 months after completion of chemo- and radiotherapy) compared to t_0 (directly prior to start of chemotherapy). In the patients treated with high-dose chemotherapy all parameters are decreased, while in the patients treated with standard-dose chemotherapy leukocytes and platelets are decreased. All parameters are lower after high-dose than after standard-dose treatment at t_1 . Mean (change of) telomere length do not differ between the groups. Telomerase activity is below detection limit in all samples. Reinfused CD34⁺ numbers (marker for hematopoietic progenitor cells), but not (change of) telomere length are related with mean corpuscular volume of the red blood cells

($p < 0.05$), leukocytes ($p < 0.001$) and platelets ($p < 0.01$) at t_1 ; CD34⁺ numbers are not related to telomere length change.

In conclusion, while following standard- and high-dose chemotherapy (in particular) the peripheral blood hematologic values initially are lower, no change of mean telomere length and/or telomerase activity is observed. Therefore, in this setting, 6 to 8 weeks after completion of chemo- and radiotherapy no support for accelerated telomere loss in hematopoietic stem cells due to hematologic proliferative stress is found.

Telomerase activity as a biomarker for (pre)neoplastic cervical disease in scrapings and frozen sections from patients with abnormal cervical smear

Telomerase activity is nowadays determined with the telomeric repeat amplification protocol (TRAP) assay, which is a polymerase chain reaction (PCR) based assay^{21,22}. Telomerase activity is almost exclusively upregulated in cancerous tissues and not or weakly expressed in normal tissue²¹. Hence, detection of telomerase activity may serve as a possible diagnostic tool in the detection of (pre)neoplastic disease. In chapter 5, the diagnostic value of semi-quantitative telomerase activity assessment in cervical scrapings together with human papillomavirus (HPV) typing for detection of preneoplastic (CIN) and neoplastic cervical lesions is examined. Subsequently, telomerase activity in cervical scrapings and frozen specimens from the same patients are compared.

Telomerase activity is detectable in assessable scrapings, however, sensitivity and negative predictive value of the TRAP assay for CIN II/III and cancer lesions are 25% and 28%, respectively, while specificity for no CIN or CIN I is 89%. In representative frozen sections, frequency of detectable telomerase activity is related to grade of CIN/cancer ($p < 0.0005$). Telomerase activity levels in paired scrapings and frozen sections appears to be only weakly related; telomerase-positive sections with telomerase-negative scrapings and vice versa (only in CIN III) are observed. In oncogenic HPV-negative scrapings, no telomerase activity is detected, but in frozen sections, telomerase activity appears to be unrelated to presence of specific HPV types.

From these data it is concluded that telomerase activity is more frequent in higher grade CIN/cancer. Telomerase activity assessment in cervical scrapings has a low sensitivity for CIN II/III and/or cervical cancer and does not appear to be useful for primary screening for cervical cancer. However, increased telomerase activity in frozen CIN sections may be a possible marker of progressive disease.

Telomerase in (pre)neoplastic cervical disease

One of the observations in chapter 5 is that telomerase activity levels in cervical scrapings are only weakly related to telomerase activity levels in frozen sections from the same cervical lesion. This frequent discrepancy may be due to heterogeneity of telomerase activity in (pre)malignant cervical lesions. Studies using *in situ* hybridisation techniques for the expression of the RNA component of telomerase, hTR, have not only shown dysregulation of telomerase activity and

hTR expression, but also important heterogeneity in hTR expression, especially in premalignant lesions of different tumour types²³⁻²⁵. In chapter 6, upregulation of hTR expression in (pre)malignant cervical lesions as determined by *in situ* hybridisation and reverse transcriptase PCR (rt-PCR) and hTERT mRNA, the catalytic subunit of telomerase, expression studied with rt-PCR are analysed. Moreover, application of *in situ* hybridisation allows the evaluation of possible heterogeneity of hTR expression within the cervical lesions.

hTR as determined by *in situ* hybridisation is related to grade of CIN/cervical cancer ($p < 0.001$). In general, hybridisation patterns for hTR expression are homogeneous throughout the lesion. hTR expression, as determined by rt-PCR, is detected in most of the cervical specimens. hTERT mRNA is related to grade of CIN/cervical cancer ($p < 0.01$). hTR expression, as determined by *in situ* hybridisation and hTERT mRNA expression by rt-PCR are related to telomerase activity levels ($p < 0.001$, $p < 0.05$, respectively) and presence of oncogenic types of HPV (both $p < 0.05$). hTR expression levels by rt-PCR shows a trend with telomerase activity levels, but not with presence of oncogenic HPV.

These data show frequent upregulation of hTR and hTERT mRNA expression in CIN lesions which appear to occur earlier than induction of telomerase activity. The fact that semi-quantitative hTERT mRNA as well as hTR levels are related to telomerase activity levels illustrates that in (pre)malignant cervical lesions upregulation of both telomerase components are important for functional telomerase.

Telomerase and clinicopathologic prognostic factors in cervical cancer

We have shown in chapter 5 and 6 that telomerase activity, hTR and hTERT mRNA expression levels are related to grade of CIN/cervical cancer. Therefore, these components may also be related to clinicopathological features. Furthermore, others have already reported that higher telomerase activity is linked to a worse prognosis in leukemias, in breast cancer and in meningiomas²⁶⁻³⁰. Therefore, in chapter 7 the potential relation between telomerase activity, hTR and/or hTERT mRNA with 'classic' clinicopathologic prognostic factors and survival in patients with cervical cancer is described. Analysis of prognostic factors and survival is limited to early stage patients, primarily treated with radical hysterectomy.

Telomerase activity is not detected in the normal cervixes and present in 79% of cervical cancers ($p < 0.001$). hTR is detected in all normal cervixes and cervical cancers. hTERT mRNA is detected in 1 of 8 normal cervixes, while 80% of cervical cancers are positive ($p < 0.001$). In contrast to semi-quantitative hTR expression levels, semi-quantitative hTERT mRNA levels are related to telomerase activity levels ($p < 0.01$). Semi-quantitative telomerase activity levels are related to differentiation grade ($p < 0.05$), but not to stage and histotype. In the early stage patients primarily treated with radical hysterectomy, semi-quantitative levels of telomerase activity are not related to tumour volume, vascular invasion or presence of metastatic lymph nodes. hTR as well as hTERT mRNA are not related to any clinicopathological factor. Tumour volume, vascular invasion and presence of metastatic lymph nodes are related to (progression free) survival, while telomerase activity or its subunits are not.

In conclusion, frequent upregulation of telomerase activity and hTERT mRNA is related to cervical cancers, while hTR is also detected in normal cervixes. Telomerase is not applicable as a prognostic factor in early stage cervical cancer patients, primarily treated with radical hysterectomy.

Telomerase in relation to expression of p53, c-Myc, estrogen receptor and progesterone receptor in ovarian tumours

In chapter 8 telomerase activity, hTR and hTERT mRNA in benign, borderline and malignant ovarian tumours are evaluated in relation to the expression of p53, c-Myc, estrogen receptor (ER) and progesterone receptor (PR). Furthermore, relations with known clinicopathologic factors as well as survival in malignant ovarian tumours are assessed.

Telomerase activity and hTERT mRNA are more frequently observed in malignant compared to borderline and benign tumours (both $p < 0.001$), whereas hTR expression is commonly detected in all types of tumours. Telomerase activity was correlated with hTERT mRNA expression ($p < 0.05$), but not with hTR expression. Immunohistochemical staining of p53 has been shown to be related to mutated p53²³². Expression of p53 and c-Myc are more frequently detected in malignant compared to borderline and benign tumours (both $p < 0.001$). Telomerase activity is positively related to p53 and c-Myc expression (both $p < 0.001$), negatively related to PR expression ($p < 0.05$), but not to ER expression. In the malignant ovarian tumours telomerase activity and hTERT mRNA expression are not related to stage, differentiation grade or residual disease. However, semi-quantitative hTR expression levels are related to stage ($p < 0.05$), but not to differentiation grade and residual disease. Stage, differentiation grade, residual tumour after first laparotomy and presence of ascites are good prognostic factors for (progression free) survival in this study. Telomerase is not related to (progression free) survival.

In conclusion, these data suggest that p53 expression (e.g. mutated p53) and c-Myc expression may be important for (up)regulation of telomerase activity.

DISCUSSION

In this thesis evaluation of telomere length and telomerase activity has been used to enlarge insight in the pathophysiological mechanisms of different human diseases, as well as to improve prediction of the course of (gynecological) malignant tumours.

Analysis of telomere length for replicative history

It has been shown that analysis of telomere length can be a good marker for replicative history of cells. Telomere length as a marker for cell turnover can only be assessed in normal cells, which contain no or weak telomerase activity. Nevertheless, it has to be taken into account that telomere length is a homeostasis, determined by cell proliferation and telomerase activity. Therefore, one should also determine the telomerase activity before drawing any conclusions on cellular ageing. Through the fact that telomere length is shortening with each cell division

and thus with age, with a large difference between individuals, it is recommended that telomere length is determined during a certain time period. Together with the telomerase activity data realistic conclusions can be drawn on the replicative history of the cells. Disadvantages of the traditional method for measuring the telomere length (Southern analysis) are that a large number of cells ($>10^5$) is required and this technique does not provide information on the telomere length of individual chromosomes. Fluorescence *in situ* hybridisation²⁴⁴ and primed *in situ* assay²⁴⁵ allow telomere length determination in individual chromosomes, while flow cytometry method using fluorescence *in situ* hybridisation allows telomere length measurement in individual cells²⁴⁶. These kind of assays may be helpful in studying which cells and/or chromosomes in premalignant tissues are beyond the critical telomere length inducing crisis.

Telomerase as diagnostic tool

Telomerase activity determination as a screening tool for cervical cancer in scrapings is not applicable. For triage of patients with mildly abnormal cervical scrapings telomerase activity determination seemed to be of modest clinical significance. However, the number of positive-telomerase scrapings from patients with a mild or moderate dysplastic smear at referral was small.

Apart from telomerase activity determination it may also be worthwhile to evaluate the value of determination of hTR and/or hTERT mRNA levels in cervical scrapings. Due to rapid degradation of telomerase proteins Müller *et al.*⁷² observed a very low sensitivity of the telomerase activity assay for detection of bladder cancer in urine samples. Using a rt-PCR assay for hTR, however, the sensitivity was increased to 85%. Rapid degradation of telomerase proteins may also be a problem in cervical scrapings and therefore determination of hTR may be more sensitive than examination of telomerase activity. However, till now no data exist on hTR and/or hTERT mRNA in cervical scrapings.

Telomerase as marker for progressive disease

In several studies in other malignancies determination of telomerase activity has been investigated for its possible use as biomarker for progressive disease. The natural history of CIN is uncertain, but progression has been presumed (and debated) of CIN I to III culminating in invasive disease. The majority of low grade CIN lesions however will regress or remain stable, with very few progressors. In contrast, it is estimated that 35-75% of high grade CIN lesions will progress to cancer over 20 years⁵⁹. In general, presence and levels of telomerase activity, hTR using *in situ* hybridisation and hTERT mRNA expression are related to grade of CIN/cancer and it is suggested that upregulation of telomerase may be an early event in cervical carcinogenesis. Thus, it may be interesting to investigate whether or not CIN III lesions positive for telomerase are in a more progressive state than their negative counterparts. However, larger longitudinal studies have to be performed without therapeutic intervention to elucidate this.

Telomerase as therapeutic target

Although the observation that telomerase can not be used as a prognostic factor in cervical, ovarian and breast cancer^{216,217}, we and others show that telomerase is strongly associated with cancer²¹. Therefore, telomerase is an interesting target for therapeutic intervention. Numerous preclinical studies have examined several ways for inhibition of telomerase through hTR-antisense treatments, which resulted in telomeric attrition, and ultimately in cell death^{9,89-98,221}. However, issues on the use of anti-telomerase agents with regard to delivery, toxicity and resistance remain to be further studied in cell lines and animal models. Due to shorter telomeres, especially premalignant and early malignant cells are the most promising targets for these therapies. However, recent observations that anti-telomerase treatment may induce apoptosis and/or may modulate sensitivity of cancer cells for chemotherapeutic drugs without relevant telomeric attrition^{89,98} present upregulated telomerase activity in malignant cells as an interesting target for a new anticancer therapy modality.

Another strategy for anticancer therapy may be the intervention in regulation of hTERT transcription as we and others show almost exclusive upregulation of hTERT mRNA in malignant tumours¹¹⁻¹³. The promoter of hTERT comprises binding sites for the transcription factor c-Myc and hormone response elements for ER and PR³⁵⁻³⁸. Intervention in inhibition of telomerase activity *in vitro* can be accomplished by treatment of cells with antisense c-Myc²³⁹ or with the anti-oestradiol tamoxifen²⁴². However, no data exist on inhibition of telomerase activity *in vivo* with this kind of treatment.

The high specificity of hTERT to malignant tissues potentially allows gene therapy with a plasmid containing a suicide gene, e.g. the herpes simplex virus gene thymidine kinase, with upstream the hTERT promoter. Through this kind of gene therapy potentially only hTERT expressing cells, e.g. the (pre)malignant cells, will die, while the not hTERT expressing cells, e.g. the normal cells, are not affected. Very preliminary results show that this gene therapy in mice resulted in regression of the tumour, while normal tissues were not affected²⁴⁷.

Whether or not this type of strategies will be successful for anticancer therapy in human has to be further elucidated.